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Report on Qiagen Columns with Precipitation versus Packed Bed Technology for Trace Amounts of DNA

E. K. Wheeler, A. M. Erler, A. Seiler

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Disclaimer

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Preface:

This report describes work funded by the Department of Homeland Security as part of the Forensics and Attribution Thrust Area in DHS' Biocountermeasures Portfolio. It is submitted to the sponsoring DHS program office as a deliverable for the FY06 Sample Management Task titled "In-Bed Purification and Amplification of Trace DNA from Contaminated Liquids" (PEP 1.2.2.14.1.7.4.3.2). The Task PI and corresponding technical author is Elizabeth Wheeler. LLNL's Sample Management program contact is David Camp.

Dr. Elizabeth Wheeler, LLNL
L-223; POB 808
Livermore, CA 94551
wheeler16@llnl.gov
925-423-6245

Dr. David W. Camp, LLNL
L-172; POB 808
Livermore, CA 94551
camp2@llnl.gov
925-423-9228

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Elizabeth Wheeler, Anne Erler, Amanda Seiler
Lawrence Livermore National Laboratory
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Executive Summary

The assured limit of detection (LOD), where 100% of the PCR assays are successful, for the Qiagen spin column is dramatically improved when combined with an ethanol precipitation step of the eluted sample. A detailed SOP for the ethanol precipitation was delivered as a separate report.[1] A key finding in the precipitation work was to incubate the ethanol precipitation at -20ºC overnight when concentrating low copy number samples. Combining this modified ethanol precipitation with the Qiagen spin columns, the limit of assured detection was improved by 1-2 orders of magnitude, for the aliquot and assay variables used. The lower limit of detection (defined as when at least 1 assay of 1 aliquot was positive) was only improved by approximately 1 order of magnitude.

The packed bed process has the potential of a 20-fold improvement in the limit of detection compared to Qiagen plus precipitation, based on a mass balance analysis for the entire DNA concentration and purification processes. Figure ES1 shows a mass balance for all the DNA processing steps. The packed bed process minimizes losses from elution, precipitation, and pipetting (aliquoting and transferring). Figure ES1 assumes that 100 copies of DNA serve as the input sample. Efficiencies for each step have been estimated based on our experiences or a worst case scenario (for example, a 50% loss was assumed for pipetting). Table ES1 summarizes the number of copies that are the input template for PCR assuming 100 copies of DNA are processed through the three options detailed in Figure ES1.

Process	Starting copies in single PCR
Packed Bed with On-Surface Amplification	90
Qiagen Spin Column	0.9
Qiagen Spin Column with Precipiation	4.5

Table ES1: Summary of theoretical number of copies of DNA into a PCR reaction based on mass balance accounting for efficiencies of each processing step, detailed in Figure 3.

Theoretically a 20-fold increase in the number of starting copies in the PCR reaction is gained when the DNA is concentrated, purified and then amplified directly on the surface of the beads in the packed bed.

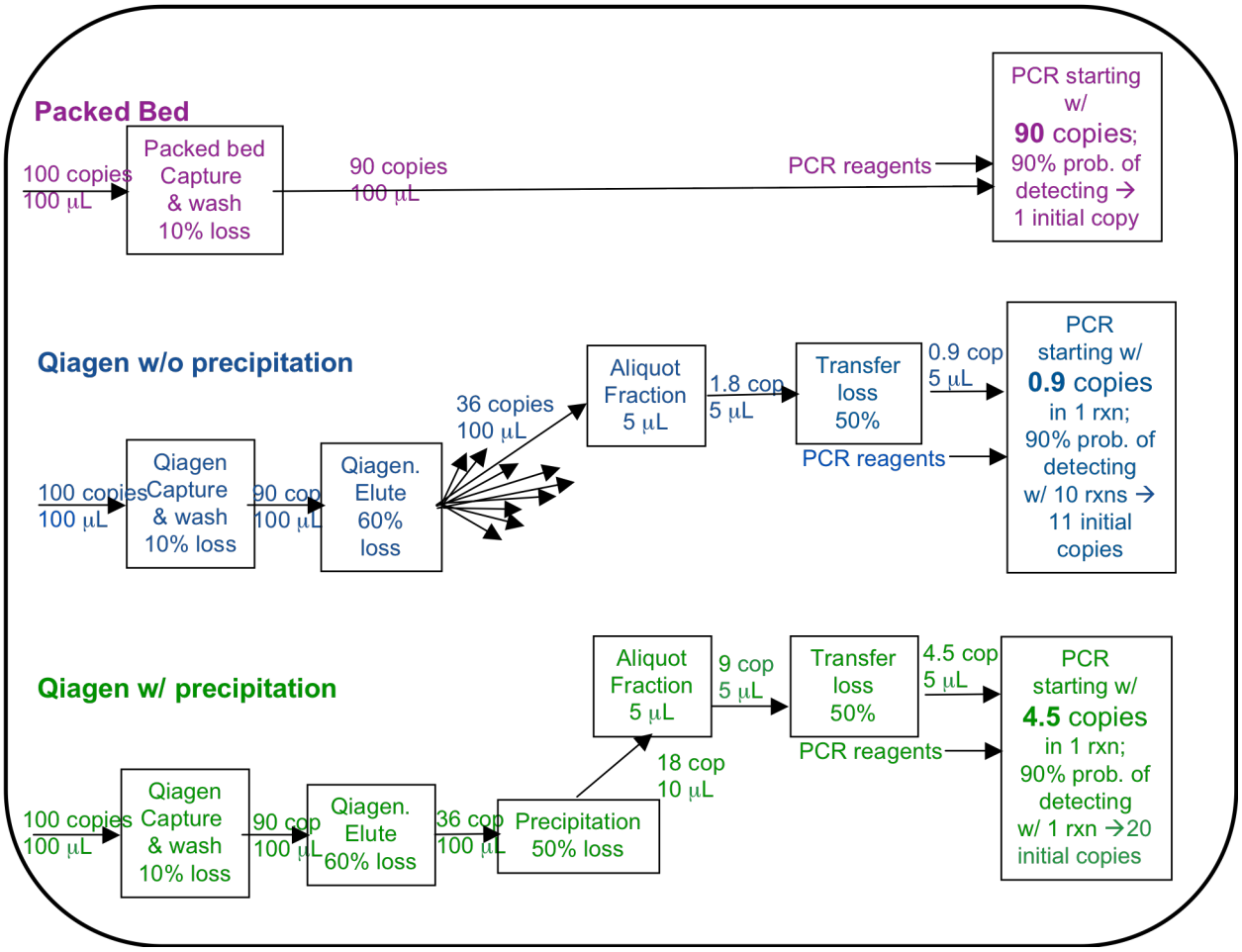


Figure ES1: Mass balance analyzing DNA losses during processing.

Background

Previous work compared the lower limits of detection (LOD) of cleaned up samples of *B. anthracis* obtained with Qiagen spin columns with those obtained from the packed bed surface amplification developed by LLNL[2]. Upon further review of these procedures, it was unclear whether the lower LODs exhibited by the packed bed technology were due to minimization of sample losses or because the entire sample, rather than an aliquot, was used during the PCR analysis.

In our previous PCR analysis, for the LLNL packed bed technology the entire bead-bound DNA sample was added to a single PCR reaction. While only 2-5 µL (2-5%) of eluted Qiagen samples were added to a single PCR reaction (only 1/50th of the sample). Although a smaller fraction of elutant was used per reaction, multiple reactions (eight) were analyzed for each Qiagen elutant. Using quantitative PCR and factoring for dilutions (ie amplifying only 2 out of 100 µL of elutant) we estimated the percentage recovery from the Qiagen columns (for various starting

concentrations). Concentrations between 10 pg and 1 ng (~10^3-10^5 genomic copies) resulted in a recovery rate of approximately 50%. For trace amounts of DNA input into the system (ie less than 1 ng), this analysis becomes more difficult due to the statistical probability of sampling the dilute target solution. To avoid this sampling probability, we chose to perform an ethanol precipitation of the 100 µL of Qiagen elutant prior to PCR.

Both of the Qiagen spin columns and LLNL's packed bed technologies for analyzing trace quantities of DNA are based on the same solid phase extraction principles. A schematic of the flow process is shown in Figure 1 with estimates of the percentage of DNA in each stream. The starting points for both the LLNL packed bed technology and the eluted Qiagen spin column are indicated in red in Figure 1. As shown in Figure 1, by not eluting the DNA off the silica surface after purification and concentration, there is a large increase (approximately 3-fold increase) in the amount of starting material for a PCR reaction.

In this project, we were asked to perform follow-up experiments to analyze the entire eluted product from the Qiagen spin columns. Upon elution from the Qiagen columns, an ethanol precipitation step was required to reduce the number of PCR reactions needed to analyze the entire sample. DNA precipitation is extremely challenging for low copy number applications.

Our previous work compared Qiagen spin columns and LLNL's packed bed methods for six different sample matrices. For this follow on task we only investigated 2 sample matrices. The procedural change to include the ethanol precipitation is shown schematically in Figure 2. After Qiagen extraction, we performed an ethanol precipitation to concentrate the eluted DNA, thus more of the Qiagen extract sample could be added to a single PCR reaction.

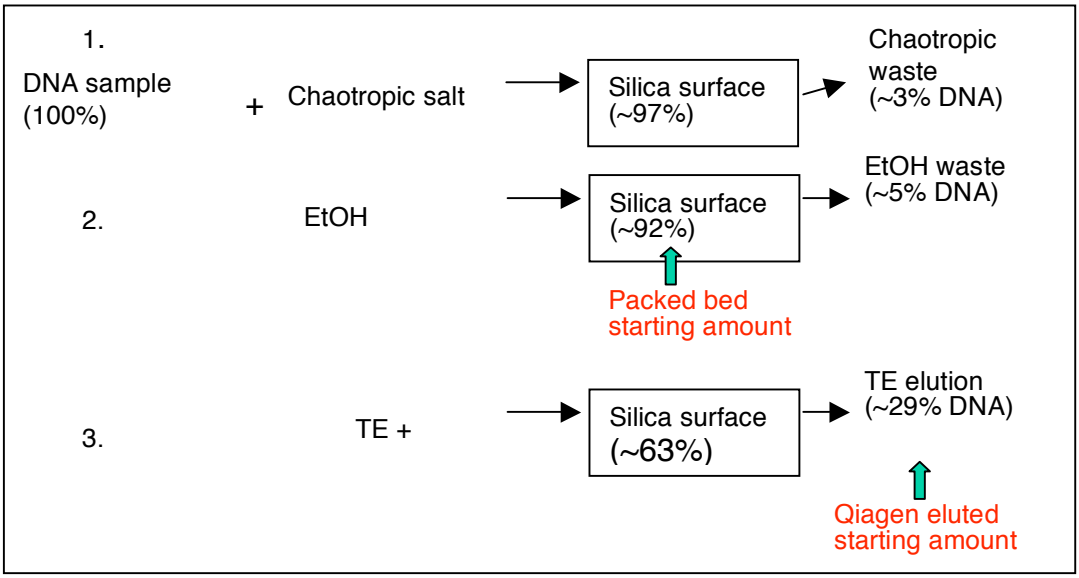


Figure 1: Flow process for solid phase extraction processes. Estimates based on early preliminary data are given for the % of input DNA in each of the different streams at the different processing steps. (Preliminary estimates from DHS funded BioBriefcase project.)

Materials and Methods

Summary of Qiagen and Precipitation Process

Figure 2 schematically summarizes the complete Qiagen with precipitation process.

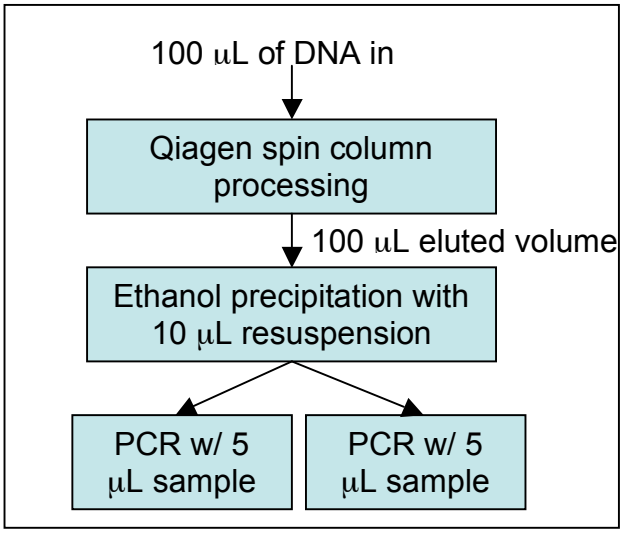


Figure 2: Standard method of Qiagen purification and elution, followed by a concentration step prior to PCR amplification..

The process for sample purification using the Qiagen spin columns is shown directly from the manufacturer's protocols in Appendix A. The spin columns used in this project were QIAamp MinElute Columns (QIAamp DNA Micro Kit (50) Cat. no. 56304; included QIAamp MinElute Columns, buffers, collection tubes).Buffers are proprietary but include: a binding buffer that contains guanidine hydrochloride, a wash buffer and an eluting buffer. The general steps include capturing the DNA on a solid phase in the column by spinning a solution of sample with the capture buffer for 1 minute at 8,000 rpm, washing matrix again with spinning, and then retrieval of the DNA by adding elution buffer and spinning at 14,000 rpm for 1 minute. PCR can then be performed on the eluted DNA.

Prior to performing precipitations, we made the following changes to the Qiagen protocol used in the previous work. Following vendor guidelines, we added carrier RNA to our initial DNA samples to aide in sample recovery. We also modified the PCR protocol to allow for additional sample volume per reaction (5 μL of sample per reaction compared to 2 μL used in the previous study).

The ethanol precipitation protocol is detailed in a separate report. In summary the steps were:

1. To 100 μL solution containing DNA
2. Add 11 μL 3 M NaOAc, 1 μL glycogen (20 μg/μL), & 275 μL ice cold 100% EtOH
3. Incubate **overnight** (17 - 20 hrs) at -20ºC
4. Centrifuged at 14,000 rpm for 30 mins at 4ºC on Beckman Coultier centrifuge
5. Aspirate EtOH
6. Wash w/ 500 μL 70% EtOH
7. Centrifuge at 14,000 rpm for 15 mins at 4ºC
8. Aspirate EtOH until the pellet is just covered
9. Air dry DNA pellet for 10 minutes
    a. To prevent PCR inhibition, DNA pellet must dry completely
10. Resuspend in 5 μL or 10 μL PCR grade water overnight (17-20 hrs)
    a. Briefly vortex to dislodge and adequately disperse the pellet throughout volume of liquid
    b. Note, smaller resuspension volumes result in more DNA loss, but higher DNA concentrations.

Packed bed process

The packed bed protocol was submitted previously (June 06) and is only summarized here in Appendix B.

Assay Procedure

After elution from the Qiagen spin columns, the aqueous DNA sample was concentrated via ethanol precipitation. The precipitated DNA sample was then resuspended in 10 μL of water. A 5 μL aliquot (50%) of sample (resuspended DNA after ethanol precipitation) was added to each PCR reaction. As in our previous report (submitted June 2006) we recorded the number of successful PCR reactions per Qiagen column. The starting mass of each sample was placed in 100 μL of water (or carpet extract) prior to processing through the Qiagen column. The sample was then concentrated to 10 μL by ethanol precipitation. The recovered sample was quantified by PCR. Ten replicates were examined for each starting concentration.

Results

Comparison of Qiagen with Precipiation to Qiagen

A comparison of the Qiagen column performance with and without precipitation is summarized in Table 1. Initial DNA samples were spiked into both water and carpet extract and processed through the clean up columns with the precipitation step. The limit of assured detection for the Qiagen columns (where 100% of the PCR reactions were successful) was improved by precipitation by 1-2 orders of magnitude when compared to the previous column data (data from Feb 06 in Table 1). The lower limit of detection (where some percentage of PCR reactions were successful) was sometimes improved by up to 2 orders of magnitude.

	Qiagen column w/ precipitation		Qiagen column (from Feb 06)	
Initial mass DNA in 100 μL	Water*	Carpet*	Water	Carpet
10 pg	10/10	10/10	9/10	3/4
1 pg	10/10	8/10	4/10	0/4
100 fg	2/10	3/10	2/10	
10 fg	0/10	1/10	0/10	

Table 1: Qiagen spin columns' LOD is improved with precipitation protocol. Data in Qiagen column without precipitation was from previous work in 2006. Results are given in # positive PCR reactions / total # PCR reactions performed.

Comparison of Packed Bed to Qiagen with Precipitation

Unfortunately, a direct head-to-head comparison of the two technologies was not possible because at the time the experiments summarized in this report were performed the packed bed technology was not performing robustly on the bench top. Results from the packed bed experiments in Feb 06 are compared to Qiagen with precipitation in Table 2.

At higher concentration, the packed bed performs equivalently or slightly worse than the Qiagen protocol with precipitation. But at trace concentrations, the packed bed performance ranges from equivalent to as much as an order of magnitude better than the Qiagen with precipitation.

	Qiagen column w/ precipitation		LLNL Packed bed (from Feb 06)	
Initial mass DNA in 100 μL	Water*	Carpet*	Water	Carpet
10 pg	10/10	10/10	8/8	8/8
1 pg	10/10	8/10	6/8	4/8
100 fg	2/10	3/10	7/8	3/8
10 fg	0/10	1/10	5/8	

Table 2: Comparison of Qiagen spin column with ethanol precipitation step and packed bed amplification on surface technology.

Discussion of Packed Bed Inconsistent Performance

A comparison of the current packed bed results to those reported earlier (2006) showed that the packed bed was clearly not performing as before. To understand this discrepancy several variables (listed below) have been investigated.

1. Different operators - due to a hiatus in funding and such a narrow scope of the follow on experiments key staff members either left the laboratory or moved on to different projects. Protocols were transferred to new team members but something may not have been adequately documented. The packed bed protocol is clearly not as robust as needed in its current documented form.

2. Degradation of DNA stocks – two different aliquots of *Bacillus anthracis* DNA that were extracted at the same time but stored separately (one having never been used until Mar 07) have been tested. No difference was seen either in the performance of the packed bed or in just PCR of dilutions of the stocks. Both aliquots of DNA yielded very similar CT values, see Appendix C. A quantitative agarose gel was also run. Analysis of the gel showed that the mass of DNA was approximately 38% lower than originally thought. Unfortunately, a similar analysis was not performed in 2006 so the quantity and quality of the DNA used previously is unknown. It is also possible that the DNA stocks from 2006 and 2007 were purified using different protocols affecting the DNA binding capability.

3. Degradation of binding reagents – fresh solutions of guanidine isothiocyante have been purchased and tested. Other chaotropic salts may yield more efficient binding.

4. Addition of RNA carrier – although not performed previously we have tried adding carrier RNA to samples that were processed through packed beds. However, the addition of carrier RNA did not improve the LOD of the packed beds.

5. Quality control of PCR reagents – amplifying DNA while bound to the surface of silica beads requires the addition of extra Taq polymerase in the PCR reaction. Experiments were previously performed varying the amount of Taq to optimize this variable. Since the lot number of the Taq used now is different from that used in 2006 the quality of the enzyme could have been slightly different, affecting the amplification in the packed bed but not the Qiagen process. A re-optimization was performed. Unfortunately, varying the amount of extra Taq per PCR reaction did not improve the LOD of the packed beds.

Based on our experimental results we hypothesize that DNA at low concentrations is not binding to the silica beads. Unfortunately, with the limited resources on this project we were unable to determine exactly which variable has changed since our earlier results. Interestingly, many of our team also worked on the BioBriefcase project that used the same technology in an automated device. The BioBriefcase successfully completed chamber testing at ECBC (Feb. 2007) and was able to detect less than 100 genome equivalents that were collected through an aerosol collector and analyzed on packed beds using the exact same protocols in an automated platform as this project.[3] Therefore, the lower limits of detection of the packed bed technology is still functioning for certain samples. This evidence indicates that understanding the starting template, whether it's extracted DNA, cells, or spores, is critical for us to generate a more robust packed bed technology that is independent of the starting material.

This data was presented to experts in PCR and solid phase extraction at LLNL in a critical review on May 9, 2007. Although this project was not able to fully understand the deterioration in the packed bed performances, the following key parameters were suggested for further investigation:

1. Change in surface chemistry of beads - determination of optimal cleaning of beads to remove any potential organics on surface that could inhibit binding.
2. Fresh aliquot of DNA that has been freshly extracted and quantified
3. Comparison of performance for various extractions of DNA and cells to better understand the importance of different parameters, such as salt content, presence of cellular debris, and other extraction variables.
4. Use reagents from Qiagen kits with packed bed – this would allow a better comparison

Conclusions

We have developed a precipitation protocol to use in conjunction with the Qiagen spin columns. We also compared commercially available Qiagen spin columns with precipitation to LLNL's packed bed technology for sample purification and concentration.

- Assured LOD for Qiagen columns with precipitation is improved 2 orders of magnitude compared to LOD for just Qiagen columns

- LOD for packed bed data (Feb 06) is approximately equivalent to the Qiagen column with precipitation
    i. Assured LOD is approximately the same for the 2 different purification and concentration processes
    ii. Lower LOD is slightly better for the packed bed technology

- Mass balance analysis suggest that packed bed technology has the potential to out perform Qiagen with precipitation by up to 20% at trace concentrations

- The packed bed technology was recently chamber tested in an autonomous platform for other projects and performed well.

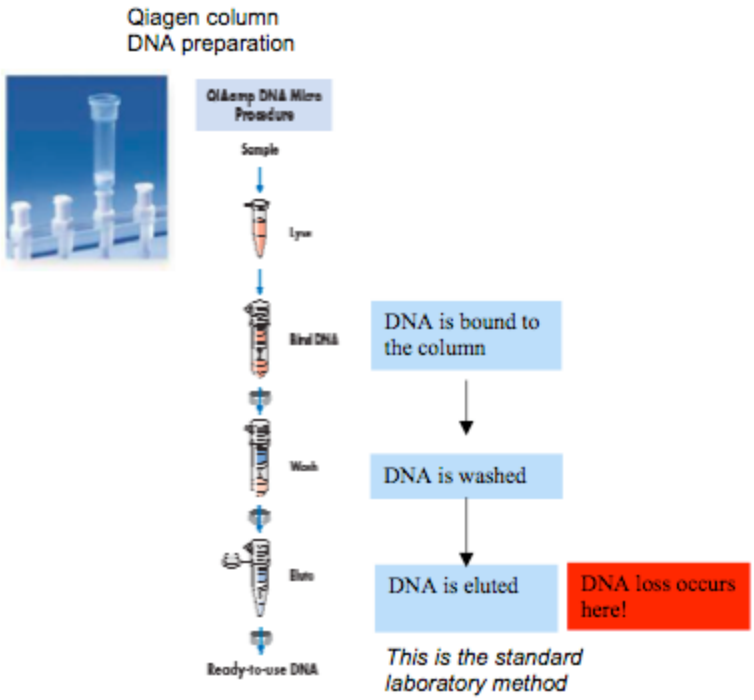
References

1. E.K. Wheeler, A.M. Erler, A. Seiler, "Precipitation report Precipitation Protocol Optimization for Trace Amounts of DNA" UCRL-TR-235840 submitted to NBFAC November 2007.

2. K. Sorensen, E. Arroyo, A.M. Erler, A.T. Chrisitan, D. Camp, E.K. Wheeler, Comparison of Packed Beds and Qiagen Columns for Recovering Trace Amounts of B. anthracis DNA from Liquid Suspensions," UCRL-TR-222387 submitted to NBFAC June 30, 2006.

3. BioBriefcase was part of DHS funded BAND program. Device was chamber tested at ECBC by their personnel for Tests 3 and 4 of BAND program. Testing completed Feb. 2007.

Appendix A: Qiagen Protocol



Appendix B: Packed bed protocol

The packed bed consists of acid washed silica beads (<106 microns) held in place in a piece of tubing by a frit. The frits used for this project were stainless steel with a porosity of 10 μm. For all experiments we used 5 mg of beads. The average size of these beads as measured by SEM was 79 μm with a standard deviation of 17 μm. Characterization of these beads showed that the DNA binding capacity saturates between 20 and 45 ng of DNA / mg of beads.

The process for using a packed bed to purify and concentrate a sample is summarized below. The key advance of this technology is Step 3, amplification of DNA directly on the beads.

Step 1, the dirty sample is mixed with a chaotropic salt / binding agents (guanidine isothiocyanate was used in this project) and introduced into the packed bed. DNA binds to the packed bed matrix.

Step 2, contaminants are washed away using ethanol.

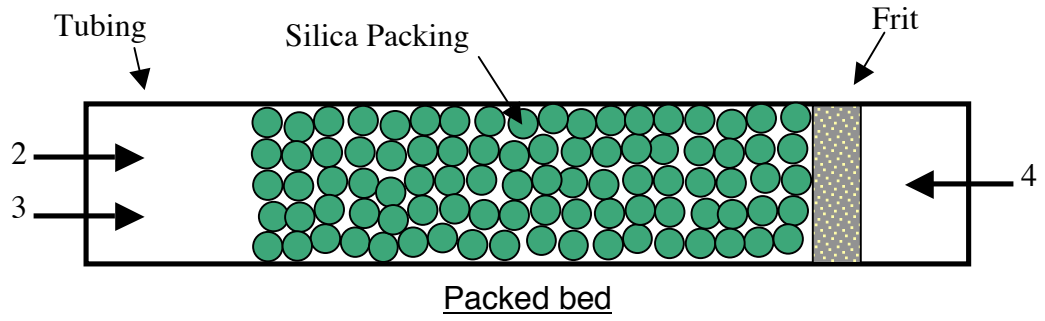
Step 3, amplification mix is introduced to the beads and thermally cycled.

Step 4, amplification markers are released for detection.

Step 5, amplified DNA is eluted from the packed bed matrix.

Procedure for using packed bed technology (details are given for flow through configuration; procedure can easily be translated for use with standard bench top equipment, if no syringe pump is available)

The schematic below shows the flow direction of steps.



1. Sample is mixed with equal volume of 6M guanidine isothiocyanate (GuSCN)
2. Sample and GuSCN mix are flowed through packed bed at 1 μl/s. The residence time in the bed is important. Note no optimization of the flow rate was performed in this project. It may be possible to process the sample faster, but the loss in capture efficiency has not been investigated. If performing on the benchtop (not flow through) then the appropriate incubation time will need to be determined.

3. Flush 1.5 mL of 70% ethanol over the beads. Flow rate is not important in this step.
4. 100 μl is back flushed through the system. Beads and ethanol slurry are collected in the Eppendorf tube that will be used for PCR amplification.
5. The ethanol supernatant is removed, and the beads are allowed to dry. Note it is important not to overdry the beads, since the DNA becomes more difficult to amplify from the surface.
6. PCR (or WGA) mix is then added to the beads from step 5.
7. The tube with beads and PCR mix is then thermally cycled on a standard PCR thermal cycler.

Appendix C: Comparison of DNA stocks